REMARKS

Claims 1, 2, 4-10, 20 and 25-31 were pending in the application. Claims 1-2 and 25- 28 have been amended. Accordingly, after the amendments presented herein have been entered, claims 1, 2, 4-10, 20 and 25-31 will remain pending. Support for the amendments to the claims can be found throughout the specification and in the claims as originally filed.

No new matter has been added. Any amendment of the claims should in no way be construed as an acquiescence to any of the Examiner's rejections and was done solely to expedite the prosecution of the application. Applicants reserve the right to pursue the claims as originally filed in this or a separate application(s).

Objection to the Drawings

The Office Action indicates that new corrected drawings are required based on the reasons set forth in the Draftsperson's comments in form PTO-948 (Paper No. 12).

Applicants submit herewith corrected drawings, and respectfully request reconsideration and withdrawal of the objection to the drawings.

Rejection of Claims 1-10, 20 and 25-31 Under 35 U.S.C. §112, First Paragraph

The Examiner has rejected claims 1-10, 20 and 25-31 under 35 U.S.C. §112, first paragraph, because, according to the Examiner, "the specification, *while being enabling for an isolated polypeptide comprising SEQ ID NO:2 encoded by the nucleic acid sequence set forth in SEQ ID NO: 1 or 3*, does not reasonably provide enablement for an isolated polypeptide or an isolated nucleic acid molecule that is at least 90% or 95% homologues to SEQ ID NO: 1, 2 or 3." (*Emphasis added*). In particular, the Examiner is of the opinion that

> [t]he specification does not provide a specific and measurable biological function or activity that can be correlated with the nucleic acid molecule Bal ... [t]he specification has not provided any indication that an increase in Bal can be correlated [to] all malignancies arising from any tissue in the body. The specification only provided the indication that a high level of Bal in a lymphoma correlates with a high risk indicating that treatment for these patients will not result in a favorable outcome.

Applicants respectfully traverse the foregoing rejection on the grounds that, based on the teachings in Applicants' specification, one of ordinary skill in the art would be able to make and use the claimed invention using only routine experimentation. Applicants wish to make clear that the instant specification teaches that elevated levels of SEQ ID NO:1 or 3 are indicative of a malignancy. Indeed, the present invention is based, at least in part, on the discovery of novel molecules, referred to herein as ***BAL nucleic acid and protein molecules which are differentially expressed in malignancies*** such as lymphoma, *e.g.*, non-Hodgkin's lymphoma (see page 7, lines 24-31 and page 9, lines 7-13 of the specification). These teachings in Applicants' specification are supported by data which demonstrates that elevated levels of SEQ ID NO:1 or 3 are indicative of a malignancy. For example, the specification discloses that "[i]n these tumors, BAL expression, as determined by the ratio of the intensity of the two co-amplified cDNAs (quantified with scanning densitometry) correlated closely with the clinical risk profile (see Figure 12). ***BAL transcripts were significantly more abundant in high intermediate/high risk primary DLB-CLs [Diffuse large B-cell lymphoma] than in cured low and low intermediate risk tumors*** (p=0.0023, Figure 12)" (see Example 3 at page 89, lines 24-31 of the specification). In addition, Example 1 teaches that

> [i]n confirmatory northern analyses, primary tumors from cured 'LR' [low-risk] patients consistently expressed low levels of BAL ***whereas tumors from 'HR' [high-risk] patients with fatal disease consistently expressed high levels of BAL (see Figure 5)***. However, only 1 of 5 DLB-CL cell lines (DHL-7) expressed high levels of BAL. This observation was of particular interest because DHL-7 grows as a semi-adherent monolayer whereas BAL-negative DLB-CL cell lines grow in suspension. These findings suggest that BAL can be upregulated when DLB-CL cells interact with other cellular or extracellular components *in vivo*. Consistent with this hypothesis, tumors derived from a DLB-CL cell line grown in SCID mice express significantly higher levels of BAL than the parental suspension cells (see Figure 6) (see page 83, lines 1-10 of the specification) ***(Emphasis added)***.

Moreover, Applicants have specifically defined the term "malignancy" to include

> a cancerous uncontrolled growth of cells in an area of the body. Malignant cancers are typically classified by their microscopic appearance and the type of tissue from which they arise. Examples of malignancies include carcinomas,

> sarcomas, myelomas, chondrosarcomas, adenosarcomas, angiosarcomas, neuroblastomas, gliomas, medulloblastomas, erythroleukemias, and myelogenous leukemias (see page 9, lines 14-19 of the specification).

Further, Applicants have disclosed in the instant specification assays for identifying all of the at least 90% or 95% homologous variants of SEQ ID NO:1 or 3 whose elevated levels are indicative of a malignancy (see, for example, pages 17, lines 23-30 and page 27, line 25 through page 29, line 5 of the specification). In particular, Applicants teach that functional allelic variants typically contain only conservative substitutions of one or more amino acids of SEQ ID NO:2 or 5, *e.g.*, a substitution, deletion or insertion of non-critical residues in non-critical regions of the protein (see page 17, lines 23-30 of the specification). Furthermore, Applicants disclose techniques for generating variants of SEQ ID NO:2 that retain functional activity of the protein (see page 27, line 25 through page 29, line 5 of the specification). In summary, it is Applicants' position that, given the guidance in the specification and the teachings in the art at the priority date of the instant application, one of ordinary skill in the art would be able to practice the invention as claimed using no more than routine experimentation.

The Examiner is also of the opinion that

> it cannot be predicted from the disclosure how to use any and all nucleic acid fragments with sequence similarity to the amino acid sequence shown in SEQ ID NO:2. Therefore, in view of the speculative nature of the invention, the lack of predictability of the prior art, the breadth of the claims and the absence of working examples, it would require undue experimentation for one skilled in the art to practice the claimed invention as claimed, which include variation in the nucleic acid sequence resulting in changes in the encoded protein sequence.

Applicants respectfully traverse the foregoing and submit that they have affirmatively taught important regions of the BLA protein, including the presence of at least one: proline rich domain (see page 10, lines 14-24 of the specification), a tyrosine phosphorylation site (see page 10, line 25 through page 11, line 7 of the specification), and a rod domain (see page 11, lines 8-17 of the specification) in the BLA protein structure. Moreover, growing databases and improved search techniques, particularly the iterated PSI-BLAST tool, has yielded substantial improvement in secondary structure prediction accuracy. Secondary structure predictions are

increasingly becoming the work horse for numerous methods aimed at predicting protein structure and function (see, for example, Koonin, E.V. *et al.*, *Curr Opin Struct Biol.* 1998 June 8(3):355-63, submitted herewith as Appendix A). Applicants thus submit that one skilled in the art could readily use the nucleic acid fragments with sequence similarity to the amino acid sequence shown in SEQ ID NO:2 as claimed using no more than routine experimentation.

The Examiner also indicates that with respect to the term "complement thereof," it is not clear if the complement thereof is a full-length complement or if this includes smaller fragments. Although Applicants traverse the foregoing rejection, in an effort to expedite prosecution and in no way conceding the validity of the Examiner's position, Applicants have amended the claims to recite "a full-length complement thereof" as suggested by the Examiner. Applicants therefore respectfully request withdrawal of the rejection under 35 U.S.C. §112, first paragraph.

Rejection of Claims 1-10, 20 and 25-31 Under 35 U.S.C. §112, First Paragraph

The Examiner has also rejected claims 1-10, 20 and 25-31 under 35 U.S.C. §112, first paragraph "as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." In particular, the Examiner is of the opinion that

> the specification has not provided any correlation between the level of Bal expression and any malignancy arising from any tissue in the body...the limitation 'wherein the elevated levels of said nucleic acid molecules are indicative of a malignancy' does not provide predictable/repeatable means of measuring a structure function relationship. Therefore, only a nucleic acid sequence of SEQ ID NO:1 or 3 encoding the polypeptide sequence of SEQ ID NO:2 meets the written description provision of 35 U.S.C. §112, first paragraph.

Applicants respectfully traverse the foregoing rejection on the grounds that there is sufficient written description in Applicants' specification regarding variants of the nucleic acid molecules and polypeptides of the invention to inform a skilled artisan that Applicants were in

possession of the claimed invention at the time the application was filed, as required by section 112, first paragraph (see M.P.E.P. 2163.02). Indeed, as set forth above, the instant specification is replete with teachings that correlate the level of Bal expression with a tumor, *e.g.*, a malignancy, as defined at page 9, lines 14-19 of the specification. Example 14 of the *Revised Interim Written Description Guidelines Training Materials* provides that a claim directed to variants of a protein having SEQ ID NO:3 "that are at least 95% identical to SEQ ID NO:3 and catalyze the reaction of A→B" with an accompanying specification that discloses a single species falling within the claimed genus, satisfies the requirements of 35 U.S.C. §112, first paragraph for written description. According to the *Guidelines*, the rational of the foregoing is that "[t]he single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which Applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO:3 which are capable of the specified catalytic activity."

Here, claims 4 and 29-31 are directed to nucleic acid molecules that are 90-95% identical to SEQ ID NOs:1 or 3 or to nucleic acid molecules encoding polypeptides that are 90-95% identical to SEQ ID NO:2, wherein elevated levels of said nucleic acid molecules or polypeptides are indicative of a malignancy. Applicants provide numerous teachings which support the disclosure that elevated levels of SEQ ID NO:1 or 3 are indicative of a malignancy. For example, Applicants teach that primary tumors from cured 'LR' [low-risk] patients consistently expressed low levels of BAL ***whereas tumors from 'HR' [high-risk] patients with fatal disease consistently expressed high levels of BAL (see Figure 5)*** (see Example 3 at page 89, lines 24-31 of the specification). In addition, Applicants have disclosed in the instant specification assays for identifying all of the at least 90% or 95% identical variants of SEQ ID NOs:1 or 3 or SEQ ID NO:4 whose elevated levels are indicative of a malignancy (see, for example, pages 17, lines 23-30 and page 27, line 25 through page 29, line 5 of the specification). Thus, based on the teachings in Applicants' specification, one of skill in the art would conclude that Applicants were in possession of the claimed invention at the time of filing.

The Examiner also indicates that with respect to the term "complement thereof," it is not clear if the complement thereof is a full-length complement or if this includes smaller fragments. Although Applicants traverse the foregoing rejection, in an effort to expedite prosecution and in no way conceding the validity of the Examiner's position, Applicants have amended the claims to recite "a full-length complement thereof" as suggested by the Examiner. Applicants therefore respectfully request withdrawal of the rejection under 35 U.S.C. §112, first paragraph.

In view of the foregoing, Applicants respectfully submit that the instant specification satisfies the requirements of 35 U.S.C. §112, first paragraph for written description and, accordingly, respectfully request that the Examiner reconsider and withdraw this rejection.

SUMMARY

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to pass this application to issue.

If a fee is due, please charge our Deposit Account No. 12-0080, under Order No. DFN-031US from which the undersigned is authorized to draw.

Dated: March 3, 2004

Respectfully submitted,

By _____

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Attachments

Beyond complete genomes: from sequence to structure and function

Eugene V Koonin*, Roman L Tatusov and Michael Y Galperin

Computer analysis of complete prokaryotic genomes shows that microbial proteins are in general highly conserved – ~70% of them contain ancient conserved regions. This allows us to delineate families of orthologs across a wide phylogenetic range and, in many cases, predict protein functions with considerable precision. Sequence database searches using newly developed, sensitive algorithms result in the unification of such orthologous families into larger superfamilies sharing common sequence motifs. For many of these superfamilies, prediction of the structural fold and specific amino acid residues involved in enzymatic catalysis is possible. Taken together, sequence and structure comparisons provide a powerful methodology that can successfully complement traditional experimental approaches.

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Current Opinion in Structural Biology 1998, 8:355–363

http://biomednet.com/elecref/0959440X00800355

Abbreviations
COGs clusters of orthologous groups
HAD haloacid dehalogenase

Introduction

The determination of the complete genome sequences of several bacteria and archea and one eukaryote [1–6,7••–12••] marked the beginning of a new age in biology. For the first time, we can take a look at the complete set of proteins present in the cells of each particular organism and try to identify the proteins responsible for each cellular function. In cases where no known proteins can be found to perform a particular task, the most likely substitutes can be predicted from the set of unassigned gene products. Clearly this can be done only by analysis of complete genomes, as partial sequences do not allow us to ascertain that certain proteins are not encoded in a given genome [13]. These new approaches are gradually changing our understanding of a variety of biological phenomena. As the number of sequenced genomes is expected to grow exponentially for the next few years, their impact on different biological disciplines will increase. We have recently discussed the implications of the complete genomes for microbial evolution [14]. Here we consider the effect of the genome revolution, together with the improving methods for sequence analysis, on our ability to predict and understand protein structure and function.

Towards a natural taxonomy of proteins and protein families

The numerous genome sequencing projects have resulted in a rapid growth of protein databases (see, e.g. [15]). In contrast to the pre-genome era, when researchers typically chose to clone and sequence genes with documented functional roles, we are now getting many protein sequences whose functions are not known. This presents a challenge to extract the most from these sequences in terms of salient features of the encoded proteins, for example to classify them according to their homologous relationships, and to predict their possible catalytic activities and/or cellular functions, three-dimensional (3D) structures and evolutionary origin.

Protein classifications, pioneered by Dayhoff and her coworkers, have historically been based on sequence alignments. Similar proteins formed families, which were combined into superfamilies [16]. This approach, continued in the PIR database [17], proved extremely popular. However, even PIR superfamilies often unite closely related proteins and more distant relationships are being missed. Other protein databases, such as PROSITE [18], PRINTS [19], Pfam [20], and ProDom [21], group proteins on the basis of conserved sequence motifs and, generally, contain much more diverse protein families. Structural comparisons of proteins, implemented in FSSP, CATH and SCOP databases, offer yet another approach to protein classification [22–24]. SCOP superfamilies, for example, unite proteins that have some similarities in their 3D structures, but often no detectable sequence similarity [25]. Thus, in the absence of clear sequence or structural similarities, the criteria for inclusion of distantly related proteins into a family (or superfamily) become increasingly arbitrary.

With the inception of extensive genome sequencing, it has become possible to classify genes and proteins on a different principle, namely by delineating families of paralogs — related genes within the same genome [26,27]. Such analyses have revealed a complex hierarchical organization of paralogous families in each of the studied genomes and produced at least two generalizations: first, the fraction of genes that belong to families of paralogs increases with the increase of the total number of genes in a genome: from ~25% in the minimal genome of *Mycoplasma genitalium* to >50% in the large (for a prokaryote) *Escherichia coli* genome; second, the largest superfamilies of paralogs are mostly the same in all genomes [28–33].

Knowledge of all the protein sequences from multiple complete genomes (Table 1) allows us to redefine the entire

Table 1

Protein families and 3D structures in complete genomes.

Species	Proteins encoded in the genome*		COGs found (% total)	3D structures‡	
	Total number	Belong to COGs† (% total)		In PDB	Predicted‡
Escherichia coli	4289	2003 (47%)	821 (95%)	240	667
Haemophilus influenzae	1717	979 (57%)	658 (77%)	2	267
Helicobacter pylori	1566	841 (54%)	617 (72%)	0	169
Synechocystis sp.	3169	1551 (49%)	796 (93%)	2	431
Borrelia burgdorferi	850	483 (57%)	363 (42%)	0	105
Bacillus subtilis	4100	1945 (47%)	732 (85%)	12	578
Mycoplasma genitalium	467	341 (75%)	290 (34%)	0	75/103
Mycoplasma pneumoniae	677	378 (56%)	309 (36%)	0	78
Methanococcus jannaschii	1715	830 (48%)	498 (58%)	0	170
Methanobacterium thermoautotrophicum	1869	897 (48%)	484 (56%)	0	199
Archaeoglobus fulgidus	2407	1131 (47%)	512 (60%)	0	290
Saccharomyces cerevisiae	5932	1736 (29%)	577 (67%)	45	846
Caenorhabditis elegans	12,178	2172 (18%)	466 (54%)	2	NA

*The numbers are from the latest updates in the GenBank genome division (ftp://ncbi.nlm.nih.gov/genbank/genomes). *C. elegans* genome is about 85% complete; the data are from Wormpep12 (www.sanger.ac.uk/Projects/C_elegans/wormpep). †Based on the set of 860 COGs, obtained by adding *H. pylori* proteins to the original set of 720 COGs [37••]. ‡The numbers are from the PEDANT database [53•], calculated by comparing the protein set encoded in each genome to the PDB using FASTA with cutoff score of 120; the second figure for *M. genitalium* is from [54•]; the data for *C. elegans* are not available.

problem of protein classification. Since the fraction of proteins conserved over large phylogenetic distances (ancient conserved domains) appears to be nearly constant at ~70% in all prokaryotic genomes [34•], it becomes feasible to replace more or less arbitrary clustering of proteins by similarity with consistent groups in which the evolutionary relationships between the members are specifically defined. Such a classification of proteins can provide a framework for evolutionary studies and for rapid, largely automatic, functional annotation of newly sequenced genomes.

Several classifications of homologous proteins encoded in complete genomes have been produced, based on all-against-all protein sequence comparisons [35,36,37••]. Each of these projects is aimed at the identification of orthologs, that is direct counterparts in different genomes, connected by an uninterrupted line of vertical descent and typically retaining their physiological function [26,27]. In particular, the system of clusters of orthologous groups (COGs) was designed to accommodate the vastly different evolution rates observed for different genes [37••]. The COGs construction procedure identifies the closest homologs in each of the sequenced genomes for each protein, even if the similarity is fairly low and not statistically significant by itself. The approach to the identification of COGs was built upon the transitivity of orthologous relationships, that is the simple notion that any group of at least three genes from distant genomes, which are more similar to each other than they are to any other genes from the same genomes, is most likely to belong to an orthologous family. Clearly, this is a probabilistic assumption based on a 'weak molecular clock concept', which posits that orthologs are more similar to each other than they are to paralogs with different, even if related, functions. This assumption, however, seems to hold true in cases where we have reasons to accept orthology on functional grounds (for example, aminoacyl-tRNA synthetases or ribosomal proteins). Orthology is not necessarily a one-to-one relationship, as in cases of lineage-specific duplications, orthology can only be established between families of paralogous genes. Such complex relationships require caution in the functional interpretation of the phylogenetic classification of proteins. Nevertheless, about 60% of the original set of 720 COGs [37••] are simple families, with no paralogs or with paralogs from one lineage only, suggesting the possibility of straightforward transfer of functional information from functionally characterized genes from model systems such as *E. coli* and yeast to those from poorly characterized genomes.

The utility of this system of protein classification was tested on several newly sequenced bacterial, archeal and eukaryotic genomes. Interestingly, with the only exception of the minimal genome of *M. genitalium*, the fraction of the proteins that belong to the COGs — ancient families conserved across a wide phylogenetic range — is about the same and very close to 50% for all prokaryotic genomes (Table 1). This is clearly compatible with the previous estimate that about 70% of the proteins encoded in each genome contain ancient conserved regions. The fraction of the proteins included in the COGs is at this time lower, which is evidently due to the requirement for three distant lineages to be included, and to the limited number of species in the first instalment of the COGs. There is little doubt that with new genomes added, the number of COGs will asymptotically approach the total number of ancient conserved regions. By contrast, this fraction is much lower

for eukaryotic genomes, indicating the prevalence of eukaryote-specific families.

Comparison of the new protein sets with the COGs resulted in a number of functional predictions for previously uncharacterized proteins. Even for the *Helicobacter pylori* proteins, most of which show highly significant similarity to homologs from *E. coli* and other bacteria and have been described in considerable detail [8••], predictions were made in more than 100 cases (http://www.ncbi.nlm.nih/COG); function was also predicted for a number of archeal and worm proteins (EV Koonin, RL Tatusov, MY Galperin, unpublished data).

Missing gene families and evolution of metabolic pathways

Comparative analysis of the available complete genomes shows that metabolic diversity generally correlates with genome size. Parasitic bacteria import a variety of metabolites, which allows them to shed genes encoding enzymes for many or even most of the metabolic pathways [1–3, 8••,33,38]. In contrast, all cells have to rely on their own gene products for performing such essential functions as genome expression, replication and repair, and membrane biogenesis and others. These tasks alone require at least about 200 genes [13,37••].

Given complete genome sequences, classification of proteins into orthologous groups provides a convenient way to systematically survey the protein families present or absent in a genome and to identify the metabolic pathways that are likely to be operative in the organism analyzed. When some of the required enzymes cannot be found in the genome, the respective pathways are either not operative, or use other, unrelated, proteins to catalyze the missing steps (see [39]). An example of such an analysis, which included superposition of the phylogenetic patterns derived from the COGs [37••], over the scheme of glycolysis, reveals several interesting trends (Figure 1). Glycolysis includes three reactions that in different species are catalyzed by non-orthologous enzymes, namely phosphofructokinases, aldolases and phosphoglycerate mutases. Interestingly, the second phosphofructokinase in *E. coli*, encoded by the *pfkB* gene, has apparently been recruited from a ubiquitous family of ribokinase-like sugar kinases. The ribokinase COG seems to be an example of a complex family in which the exact orthologous connections are not always easy to trace. In particular, even though PfkB formally belongs to the COG, there seems to be no actual ortholog of it in other genomes. Thus *H. pylori* does not encode a phosphofructokinase at all, although it has genes for other kinases of the ribokinase family and, accordingly, is represented in the respective COG (Figure 1).

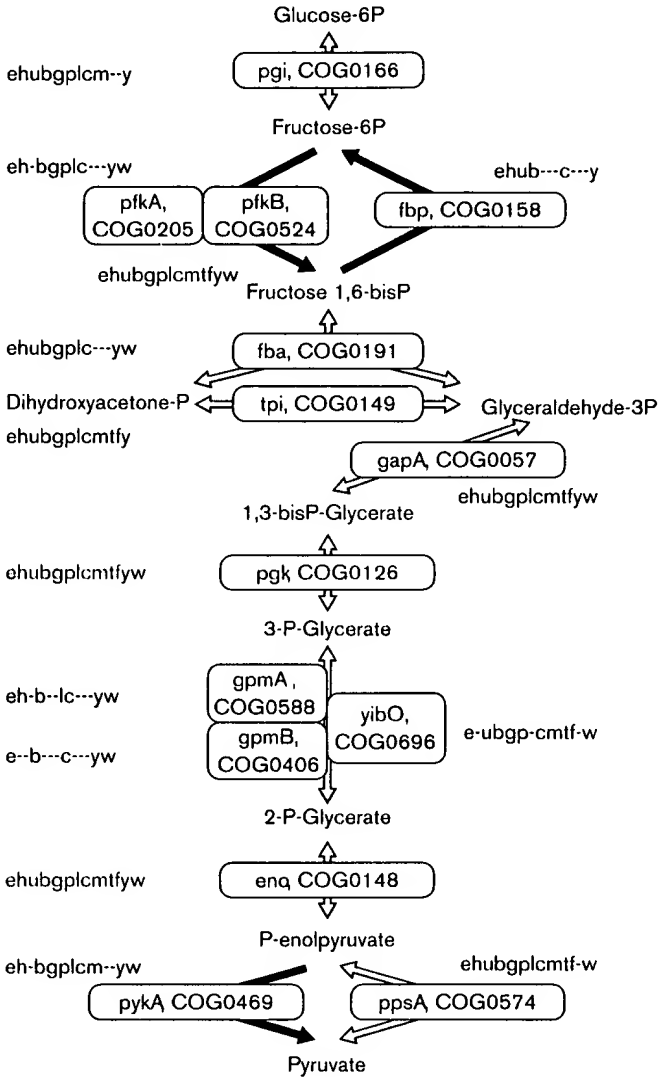
A remarkable case of non-orthologous gene displacement involves two unrelated forms of phosphoglycerate mutase, the 2,3-bisphosphoglycerate (BPG)-dependent and the BPG-independent one. While *H. influenzae* and *Borrelia burgdorferi* encode only the BPG-dependent form, and *H. pylori*, mycoplasmas, and archea encode only the BPG-independent form (see [40]), free-living bacteria such as *E. coli*, *Bacillus subtilis* and *Synechocystis* sp. possess genes coding for both these forms, with two paralogs of the BPG-dependent one (Figure 1). Phosphofructokinase, aldolase and fructose bisphosphatase genes are all missing in the archea (Figure 1), in accordance with the experimental data [41]. This is consistent with the idea that glycolysis originally evolved as a biosynthetic pathway, containing only the lower (tri-carbon) part [42].

Systematic identification of missing links in functional systems in organisms for which complete genome sequences are available is probably the most important application of protein family classification. Conspicuous gaps in the *H. pylori* metabolism became apparent from the COG analysis, suggesting major revisions to the general scheme of the central metabolic pathways in this bacterium (Table 2). In particular, unlike most other bacteria (and all with completely sequenced genomes), *H. pylori* seems to possess neither glycolysis nor the pentose phosphate shunt, the Entner-Doudoroff pathway being the only major route of sugar catabolism. Indeed, sugar fermentation, resulting in intracellular acid production, would be an additional burden on the pH maintenance mechanism in this bacterium, which has to survive in an external pH of 2–3. By contrast, gluconeogenesis, which converts organic acids into sugars required for nucleic acid and peptidoglycan biosynthesis and thus removes H^+ from the cytoplasm, appears to be fully functional in *H. pylori*. For the purpose of energy production, *H. pylori* apparently depends on amino acid fermentation, which causes alkalinization of the cytoplasm and thus relieves part of the problem of pH maintenance. Amino acids and oligopeptides that serve as substrates for this fermentation are produced by gastric proteolysis and transported by readily identifiable permeases.

From genomes and families to superfamilies and folds

Classification systems aimed at the identification of families of orthologs make no attempt to capture the more subtle conserved motifs in proteins, which reflect ancient relationships at the level of superfamilies and frequently are critically important for understanding protein functions and structures [43,44]. Computer methods for the detection of such motifs and delineation of superfamilies have lately progressed significantly through programs such as BLIMPS/MULTIMAT [45], Probe [46], and PSI-BLAST [47••], which combine pairwise sequence comparisons with profile analysis. PSI-BLAST, in particular, has proved to be a powerful tool for the detection of subtle sequence motifs, resulting in the discovery of a number of unsuspected superfamily relationships [47••,48•]. Furthermore, one of the perhaps under-appreciated benefits of the accumulation of genomic sequences is the greatly improved capacity to identify even very subtle sequence similarities due to

Figure 1



Glycolytic enzymes in organisms with completely sequenced genomes. The enzymes are listed under *E. coli* gene names. The COG numbers are as in COG database (www.ncbi.nlm.nih.gov/COG, [37••]) (where available). Shaded arrows indicate reversible reactions, black arrows practically irreversible ones. Phosphoenolpyruvate synthase-catalyzed reaction in the direction of phosphoenolpyruvate hydrolysis has been demonstrated *in vitro*. Phylogenetic patterns are: e, *Escherichia coli*; h, *Haemophilus influenzae*; u, *Helicobacter pylori*; b, *Bacillus subtilis*; g, *Mycoplasma genitalium*; p, *Mycoplasma pneumoniae*; l, *Borrelia burgdorferi*; c, *Synechocystis* sp.; m, *Methanococcus jannaschii*; t, *Methanobacterium thermoautotrophicum*; f, *Archaeoglobus fulgidus*; y, *Saccharomyces cerevisiae*; w, *Caenorhabditis elegans*.

the increasingly uniform population of the protein universe by these relatively unbiased sequence sets, of which the new methods for sequence analysis mentioned above can take advantage [49•].

In the past year, we have seen the identification or significant extension of a number of protein superfamilies; some examples, with the distribution among complete genomes, are shown in Table 3. Most of these superfamilies are universally found in all genomes, with the counts more or less proportional to the total number of genes in the genome. Some expansions are, however, remarkable, such as, for example, urease-related hydrolases and ATP-grasp domains in the archea, and HAD superfamily hydrolases in *E. coli* and *B. subtilis* (Table 3). In certain cases, the phylogenetic distribution of a superfamily immediately suggests major evolutionary events. Thus the BRCT domain is present in a single copy in the DNA ligase of all bacteria (with one additional copy found only in *Synechocystis*), is missing in the archea, and is dramatically expanded in its distribution in the eukaryotes (Table 3). The most obvious interpretation of this distribution is that this domain has entered the eukaryotic world by horizontal gene transfer from bacteria and has undergone exten-

Table 2

Genes and pathways missing in *Helicobacter pylori*.

Enzyme activity	*E. coli* gene	COG number	Status in *H. pylori*	Implications for *H. pylori* metabolism
Phosphofructokinase	*pfkA*	COG0206	Missing	Absence of the two key glycolytic enzymes shows that Embden-Meyerhof pathway is not functional in *H. pylori*. Gluconeogenesis enzymes, bypassing these reactions, fructose bisphosphatase (HP1385) and phosphoenolpyruvate synthase (HP0121), are present in *H. pylori*, allowing it to produce sugars required for peptidoglycan biosynthesis.
	pfkB	COG0525	Present (ribokinase)	
Pyruvate kinase	*pykA*	COG0470	Missing	
	pykF			
6-phosphogluconate dehydrogenase	*gnd*	COG0360	Missing	Pentose phosphate pathway is also not functional. Even though *H. pylori* has a ribose 5-phosphate isomerase encoded by an ortholog of the E. coli *rpiB*, no gene coding for 6-phosphogluconate dehydrogenase could be identified. The only saccharolytic pathway in *H. pylori* appears to be the Entner-Doudoroff pathway.
Ribose 5-phosphate isomerase	*rpiA*	COG0120	Missing	
Lipoate synthase	*lipA*	COG0318	Missing	Pyruvate dehydrogenase complex is absent in *H. pylori*; acetate kinase and phosphotransacetylase are not functional. Pyruvate-ferredoxin oxidoreductase is the only acetyl-CoA-producing enzyme in *H. pylori*.
Lipoate-protein	*lplA*	COG0411	Missing	
ligase	*lipB*	COG0319	Missing	
Dihydrolipoamide acyltransferase	*aceF*	COG0510	Missing	
Acetate kinase	*ackA*	COG0280	Disrupted by a frameshift	
Phospho-transacetylase	*pta*	COG0278	Disrupted by frameshifts	
Enzymes of purine biosynthesis	*purF*	COG0034	Missing	*De novo* purine biosynthesis is absent in *H. pylori*, and it has to obtain purines from the host. HP1185 appears to be the best candidate for the purine permease, as it is the only *H. pylori* protein, similar to *E. coli* PurP.
	purD	COG0151	Inactivated by mutations	
	purN	COG0299	Missing	
	purT	COG0027	Missing	
	purL_1	COG0046	Missing	
	purL_2	COG0047	Missing	On the other hand, *H. pylori* encodes the enzymes for AMP and GMP synthesis from IMP and their interconversion. Therefore, it can survive on any of these purines.
	purM	COG0150	Missing	
	purK	COG0026	Missing	
	purE	COG0041	Missing	
	purC	COG0152	Missing	
	purH	COG0138	Missing	
	purA	COG0104	Present	
	purB	COG0015	Present	
	guaB	COG0516	Present	
	guaA_1	COG0518	Present	
	guaA_2	COG0519	Present	

sive duplication with divergence in the eukaryotes. The expansion of this domain into a number of eukaryotic proteins involved in cell-cycle control [50••,51] may have been critical for the very establishment of these systems.

With the current acceleration in protein structure determination [22,24], a superfamily identified by sequence comparison more and more frequently extends to include proteins with known 3D structure and/or well-characterized catalytic mechanism (Table 3). Such findings are sometimes most illuminating as they immediately result in the prediction of the structural fold, the structure of the active center, and possibly also the catalytic mechanism for a wide variety of diverse proteins comprising the superfamily. This is illustrated by the recent prediction of the structure and the catalytic amino acid residues for P-ATPases, which remained elusive in spite of a long history of studies, on the basis of the sequence motifs shared with haloacid dehalogenases [52•].

Assignment of the gene products to structural folds and families with maximal attainable precision is arguably one of the foremost tasks of genome analysis after the sequencing phase. The number of structures that have been determined experimentally is negligible for almost all genomes, with the exception of *E. coli* (where it is still rather a small fraction) (Table 1). A database search with a deliberately conservative similarity cut-off already increases the fraction of proteins for which a confident structure prediction is possible to 10–25% [53•] (Table 1). Secondary structure-based threading allows

Table 3

Some recently identified or significantly expanded protein superfamilies.

Superfamily	Enzymes with known 3D structures (PDB codes)	Enzymes with newly predicted properties	Representatives in complete genomes*	References
BRCT (conserved domain in cell cycle checkpoint proteins)	None	DNA polymerase subunit DPB11, terminal deoxynucleotidyltransferases, deoxycytidyl transferase, DNA ligases III and IV, poly(ADP-ribose) polymerase	e-1, h-1, u-1, b-1, g-1, p-1, l-1, c-2, m-0, t-0, f-0, y-9, w-8	[50]
Urease-related metal-dependent hydrolases	Urease (2kauC), phosphotriesterase (1pta), adenosine deaminase (1fkx)	AMP deaminase, adenine deaminase, cytosine deaminase, hydantoinase, dihydroorotase, allantoinase, aminoacylase, imidazolonepropionase, arylphosphatase, chlorohydrolase, formylmethanofuran dehydrogenase	e-13, h-4, u-2, b-6, g-1, p-1, l-1, c-3, m-9, t-10, f-7, y-6, w-9	[55]
Acid phosphatases	Vanadium-containing chloroperoxidase (1vnc)	Phosphatidic acid phosphatase, phosphatidylglycerol phosphatase, diacylglycerol pyrophosphate phosphatase, glucose-6-phosphatase	e-4, h-1, u-3, b-2, g-0, p-0, l-0, c-1, m-3, t-1, f-0, y-7, w-4	[56,57]
ATP-grasp (ATP-dependent C-N and C-S ligases)	Glutathione synthetase (1gsh), D-ala-D-ala ligase (2dln), biotin carboxylase (1bnc), carbamoyl phosphate synthase (1jdb), succinyl-CoA synthetase (1scu)	Phosphoribosylamine-glycine ligase, phosphoribosylglycinamide formyltransferase, phosphoribosylaminoimidazole carboxylase, tubulin-tyrosine ligase, protein S6-glutamate ligase (RimK), malate thiokinase, ATP-citrate lyase	e-10, h-5,u-4, b-12, g-2,p-2, l-1, c-7, m-9, t-8, f-11, y-10, w-15	[58]
HAD (phosphatases and other hydrolases)	L-Haloacid dehalogenase (1jud)	Phosphoserine phosphatase, phosphoglycolate phosphatase, histidinol phosphatase, glycerol-3-phosphatase, sucrose phosphate synthase, phosphomannomutase, P-type cation-transport ATPases	e-10, h-3, u-1, b-11, g-4, p-5, l-2, c-8, m-3, t-4, f-3, y-9, w-6	[52]
DHH (hydrolases)	None	Exopolyphosphatase, 5′-3′ exonuclease	e-1, h-1, u-4, b-6, g-2, p-2, l-2, c-3, m-6, t-3, f-7, y-1, w-0	[59]
Alkaline phosphatase-related metal-dependent hydrolases	Alkaline phosphatase (1alk), N-acetylgalactosamine 4-sulfatase (1fsu), cerebroside sulfatase(1auk)	Phosphopentomutase, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, streptomycin-6-phosphatase, phosphonoacetate hydrolase, phosphoglycerol transferase, nucleotide pyrophosphatase, steroid sulfatase, aryl- and hexosamine sulfatases	e-15, h-5, u-4, b-8, g-1, p-1, l-0, c-1, m-2, t-2, f-3, y-6, w-18	[40,60]

*Identified by PSI-BLAST [47••] searches of the complete genomes using the conserved motif(s) for each superfamily as a query. Organism abbreviations are as in Figure 1: e, *E. coli*; h, *H. influenzae*; u, *H. pylori*; b, *B. subtilis*; g, *M. genitalium*; p, *M. pneumoniae*; l, *B. burgdorferi*; c, *Synechocystis sp.*; m, *M. jannaschii*; t, *M. thermoautotrophicum*; f, *A. fulgidus*; y, *S. cerevisiae*; w, *C. elegans*.

another relatively small but notable increase in the predictive power [54•] (Table 1). It appears, however, that at this time, the most realistic way to further structure prediction at genome scale is to perform a complete analysis of protein superfamilies as exemplified in Table 3.

Perspective

As far as prokaryotic genomes are concerned, we have already entered the post-genomic era. While surprises certainly wait ahead, there is little doubt that the major protein families are already known or can be deciphered from the available sequences. We have recently seen major progress in methods and procedures for advanced sequence analysis, and a lot of valuable information has been extracted from the genomes. We believe, however, that a major focused effort in genome comparison is still required in order to construct a proper classification of protein families and superfamilies and systematically apply it to the goals of structural and functional prediction. Such an effort will have the potential of creating a basis for a rationally designed, decisive onslaught on structure determination and experimental identification of gene functions using computer predictions as a guide. Hopefully, this research program turns out to be both realistic and efficient.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

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- •• of outstanding interest

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The completion of the genome sequence of *E. coli*, one of the classic objects of molecular biology and genetics, certainly has a symbolic significance. More importantly, the enormous amount of information available regarding *E. coli* gene functions can now be used to full potential for inferring functions of homologs in other species. However, the functions of about one half of the *E. coli* genes have not been determined experimentally, and so there is still a lot to learn about *E. coli* itself.

8. •• Tomb J, White O, Kerlavage A, Clayton R, Sutton G, Fleishmann R, Ketchum K, Klenk HP, Gill S. Dougherty BA *et al.*: **The complete genome sequence of the gastric pathogen *Helicobacter pylori*.** *Nature* 1997, **388**:539-547.

The genome sequence of this bacterium is of special interest from several points of view. The genome analysis will have important practical implications as *H. pylori* is the causative agent of peptic ulcers and is believed to infect up to half of the human population. *H. pylori* thrives in a highly acidic environment (pH 2–3); deciphering the mechanisms of acid tolerance from the genome sequence is a most interesting task. Furthermore, *H. pylori* represents an early branching of the proteobacterial lineage, and the comparison of its genome with those of other Proteobacteria such as *E. coli* and *Haemophilus influenzae* will shed light on the evolution of cellular functions in bacteria and mitochondria.

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This second genome of a methanogenic archeon to be sequenced, after *Methanococcus jannaschii*, is of major importance in corroborating trends revealed by the *M. jannaschii* genome analysis [4,34]. Like *M. jannaschii*, there is a sharp divide between the majority of the genes, which appear to have bacterial origin, and a minority (primarily encoding proteins involved in genome replication and expression) of 'eukaryotic' genes. Some other unusual aspects of the *M. jannaschii* genome, however, did not recur in *M. thermoautrophicum*. For example, unlike *M. jannaschii*, *M. thermoautrophicum* encodes a typical set of molecular chaperones such as DnaK and DnaJ and does not encode a unique ATPase family found in *M. jannaschii*.

10. •• Klenk HP, Clayton RA, Tomb JF, White O, Nelson KE, Ketchum KA, Dodson RJ, Gwinn M, Hickey EK, Peterson JD *et al.*: **The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*.** *Nature* 1997, **390**:364-370.

The first sequence of a non-methanogenic archeon, and the third complete archeal genome altogether. With 2436 genes, the *A. fulgidus* genome is considerably larger than those of *M. jannaschii* and *M. thermoautrophicum*, in part due to more extensive duplication in some of the gene families. Unlike *M. jannaschii* and *M. thermoautrophicum*, *A. fulgidus* does not seem to encode any inteins. With three genome sequences available, there is for the first time an opportunity for an informative comparative analysis of archeal genomes. Definitive work in this area remains to be done, but it is already clear that the three genomes generally are highly coherent, and also that there are many mysterious conserved families, creating a challenge for further research, both theoretical and experimental.

11. •• Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni G, Azevedo V, Bertero MG, Bessieres P, Bolotin A, Borchert S *et al.*: **The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*.** *Nature* 1997, **390**:249-256.

The second classic bacterial model, after *E. coli*, and also the second largest bacterial genome sequenced so far (4100 genes compared with 4288 genes in *E. coli*). With *B. subtilis* adequately representing the Gram-positive lineage (only the minimal genomes of *Mycoplasma* had been available before), we may now have a sampling of the great majority of bacterial gene families. In addition to its value for comparative analysis, *B. subtilis* is most interesting and important in its own right, given, for example the large number of genes in its genome that encode enzymes of secondary metabolite synthesis.

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A major revamp of BLAST, which is definitely the most popular current method for database search. The key innovations are: first, the program now makes gapped alignments, with appropriately modified statistics, which results in significant increase of sensitivity; and second, the associated program PSI (Position-Specific Iterating)-BLAST makes a position-specific weight matrix (profile) out of the first pass results and iterates searches with this profile until no new sequences with similarity scores above a defined cut-off are detected. This appears to be the most powerful existing method for detection of subtle similarities between protein sequences and delineation of protein superfamilies.

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